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Genetic variation in Acremonium coenophialum (Morgan-Jones and Gams) /

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GENETIC VARIATION IN *Acremonium coenophialum*

(Morgan-Jones and Gams)

A Thesis Presented

by

HONGCHUAN LIU

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ABSTRACT

GENETIC VARIATION IN Acremonium coenophialum

(Morgan-Jones and Gams)

MAY 1993

HONGCHUAN LIU, B.S., BEIJING AGRICULTURAL UNIVERSITY

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Directed by: Dr. William A. Torello

The fungal endophytes in the grasses of tall fescue (*Festuca arundinacea*) and *Poa autumnalis* are considered to be *Acremonium coenophialum*. These endophytes grow intercellularly in their hosts and reproduce through seed without causing any visible symptoms. The endophyte infected grasses have greater insects resistance, more efficient nitrogen utilization, drought tolerance, high regrowth rates, and they are larger and more competitive in mixtures with noninfected plants. *Acremonium coenophialum* is, therefore, considered a potential biocontrol agent. The noted variation of *Acremonium coenophialum* with regard to growth rate, production of ergot alkaloids and isozyme pattern suggested that there is genetic variation within *Acremonium coenophialum*. In this research, the genetic variation among *Acremonium coenophialum* endophytes from four

host grasses was studied by Random Amplified Polymorphic DNA analysis. Only slight variation were found among the *Acremonium coenophialum* in 'Titan' 'Rebel II' and 'Shenandoah' tall fescue cultures. The *Acremonium* from *Poa autumnalis* was, however, significantly different from *Acremonium coenophialum* from tall fescue.

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LITERATURE REVIEW

A. Significant

To avoid potential problems with chemical herbicides and insecticides, biocontrol agents have been given significant more attention. The association between fungal endophytes and their host grasses reveal that fungal endophytes can increase overall vigor, insect resistance and stress tolerance of their hosts. Fungal endophytes remain within their hosts so that their hosts can continue to benefit through generations. Endophytes of grasses have strong potential as biocontrol agents, which can be economical and environmentally safe. Accurate identification of fungal endophytes can help to select endophyte which retain increased beneficial characteristics (such as vigor and resistance) and fewer unwanted characteristics (such as livestock toxicity).

B. Endophyte Classification

The association between grasses and fungal endophytes is considered to be the source of toxin causing a condition

in cattle and sheep known as "ryegrass staggers" (Fletcher and Harvey, 1981). Recently, however, endophytes have been considered potential biocontrol agents for numerous insect pests such as the Argentine stem weevil (Prestige, 1985), fall armyworm and hair chinch bug (Cheplick and Clay, 1988; Johnson-Cicalise and White, 1990).

Three plant families (Poaceae, Cyperaceae and Juncaeae) are known to have fungal endophytes (Clay 1989). Most of the endophytic fungi of grasses belong to the tribe Balansiae, in the family Clavicipitaceae. There are five genera (about 30 species) in this tribe: *Atkinsonella*, *Myriogenospora*, *Balansia*, *Balansiopsis* and *Epichloe* (Diehl, 1950; Luttrell and Bacon, 1977). Many Balansiae endophytes sporulate on their hosts, produce visible signs of infection and cause sterilization of their hosts (White, 1988). In addition to the above mentioned teleomorphic species, there are anamorphic forms. For example, *Acremonium typhinum* is the anamorph of *Epichloe typhina* (Morgan-Jones and Gams, 1982; Latch et al., 1984). *Acremonium* (*Acremonium coenophialum* and *Acremonium lolii*), from tall fescue (*Festuca arundinacea*) and perennial ryegrass (*Lolium perenne*) respectively, do not produce choke on their hosts and therefor are not disseminated by spores. They are transmitted maternally (Morgan-Jones and Gams, 1982; Rekard et al., 1985; Siegel et al., 1987; Sampson, 1933; and White and Cole, 1985). Because

Acremonium does not produce symptoms on their hosts or suppress flowering and can be inherited maternally from generation to generation, they offer the greatest potential for exploitation as biocontrol agents (Clay, 1989).

C. Acremonium as a Biocontrol Agents

The association between host grasses and their fungal endophytes is highly significant. The endophytes can derive nutrients, photosynthates, and a means of dissemination from their host plants (Smith et al., 1985). On the other hand, because clavicipitaceous endophytes can produce biologically active compounds such as ergot alkaloids and loline alkaloids (Bacon et al., 1986 and Lyons et al., 1986), endophyte infected grasses exhibit greater insect and disease resistance than endophyte free grasses (Funk et al., 1983 and 1985). Additionally, endophyte infected grasses usually have greater stress tolerance and increased overall vigor (Bates and Joost, 1990; Clay 1987a; Lyons et al., 1990; Pinkerton et al., 1990).

Most endophytes are spread among host grasses by production of conidia and ascospore, but *Acremonium* species, which can not produce conidia or ascospore on their host, are disseminated through the host seed (Clay 1986, 1988 and

1989; Siegel et al., 1987a). Except for several species, most species occur vegetatively as convoluted intercellular hyphae running parallel to the long axis of host cells in leaf and stem tissue (Clay, 1989). In the spore-producing stage, hyphae reach external areas of the host to form stromata at the time of host flowering. On the other hand, the mycelium of *Acremonium* grow intercellularly into the ovule of the host and are dispersed via the host seeds (Siegel et al., 1987b). During dormancy, the endophytes are associated with the aleurone layer of the seeds, and then invade the endosperm and infect the developing leaf sheath after germination of the seed (Siegel et al., 1985 and 1987b; Philipson and Christy, 1986).

Production of alkaloids in fungal endophyte is thought to be the chemical basis of many agronomic characteristics of the host-endophyte association. Ergot alkaloids produced by the seed-borne endophyte *Acremonium coenophialum* show physiological activities against mammals and insects. They caused the symptoms of mammalian toxicosis, and antifeeding and antibiosis effects on the fall armyworm (Clay and Cheplick, 1989; Jonson et al., 1985). Peramine and lolitrems produced by *Acremonium lolii* endophytes of ryegrass, which do not produce ergot alkaloids, show antiherbivore properties (Rowan and Gaynor, 1986). Additionally, both *Acremonium coenophialum* and *Acremonium lolii* can produce

loline alkaloids (Jones et al., 1983 and Siegel et al., 1987a) which cause reduced feeding and weight gain on fall armyworm, European cornborer and a variety of other insects (Riedell et al., 1990).

By producing alkaloids, *Acremonium* endophytes cause many syndromes on mammals, including decreased weight gain, muscle spasms, lowered milk production, gangrene, vasoconstriction and abortions (Bacon et al., 1986; Hoveland et al., 1983; and Seman et al., 1990). Some endophytes also inhibit cellulose digestion and produce volatile fatty acid production (Kennedy and Bush, 1983; Bush et al., 1982).

Resistance to insects is an important feature in the association between endophytes and their hosts. Because the alkaloids produced by endophytes are extremely toxic to insects (Clay and Cheplick, 1988; and Johnson et al., 1985), endophyte infected grasses show strong resistance to insects. Endophyte infected grasses can reduce the survival and weight gain of fall armyworm, hairy chinch bug and four species of billbug (Clay et al., 1985; Rowan and Gaynor, 1986; Cheokucj and Clay, 1988; and Johnson-Cicalese and White, 1990). Hairy chinch bugs and many other insects show strong preferences for the endophyte free grasses and tissues other than those of endophyte infected (Johnson et al., 1985; Mathias et al., 1990; and Siegel et al., 1985).

Therefor the endophyte-infected grasses have antibiosis effects.

When the endophyte hyphae is in the meristematic regions of the host may influence hormonal regulation of cell differentiation and development leading to increased vegetative growth and vigor. Endophyte can synthesize plant growth regulators, such as 3-indole acetic acid, 3-indole ethanol, 3-indole acetamide and methyl-3-indole carboxylate (Porter et al., 1985; and Bacon, 1985), and the biosynthesis of both ergot and 3-substituted indolyl alkaloids may be regulated by catabolism of host tryptophan (Porter et al. 1985). The endophyte infected grasses shows more nitrogen utilization (Lysons et al., 1986 and 1990) and high regrowth rate. Plants infected with endophytes are also capable of changing the osmotic potential of the cell sap to adapt to drought conditions (Elmi et al., 1989, Arachevalata et al., 1989; Belesky et al., 1987; Siegel et al., 1987; and West et al., 1987). Endophyte-infected plants are larger and, generally, more competitive in mixtures with non-infected plants (Hill et al. 1990).

In vitro experiments have shown that edophyted infected grasses may have antifungal activity against a number of plants pathogens. The isolates of *Acremonium coenophialum* and many other endophytes inhibited the growth of *Nigrospora*

sphaerica, *Phoma sorghina* and *Rhizoctonia cerealis* (White and Cole, 1985) and twelve grass pathogens (Siegel and Latch, 1991).

Overall, fungal endophytes, especially the *Acremonium*, show great potential of biocontrol agents in the following main areas: 1) Increasing the growth, vigor and competition ability of host plants; 2) Resistance to insects; 3) Antifungal activities; 4) Reproduction through maternal transmission of the host seeds.

D. Variation of Endophytes

The classification of *Acremonium* is based on morphological characters of mycelium and spores in culture (Morgan-Jones and Gams, 1982). Variation among the *Acremonium coenophialum* isolates has been noticed for a long time. *Acremonium coenophialum* on semi-solid medium varied in their grow rates. In liquid culture, *Acremonium coenophialum* shows different phenotypes. The fungal balls formed by the mycelium isolated from *Poa autumnalis* are much larger than those from 'Titan' and 'Shenandoah' tall fescue (Noble, 1992). Alkaloid production by the tall fescue endophytes and other clavicipitaceous endophytes of grasses has been shown to vary considerably among isolates (Bacon, 1988; Bacon and

Siegel, 1988; Porter et al., 1979). Biochemical studies have shown that *Acremonium coenophialum* endophytes exhibit variation of isozyme patterns among isolates from different host species (Leuchtmann and Clay, 1990). Among the 52 studied isolates of *Acremonium coenophialum* from tall fescue, 47 had the same isozyme phenotype except five isolates from cultivar 'Triumph'. The *Acremonium coenophialum* endophyte isolated from *Poa autumnalis* also showed a significant differences. These studies indicate that there might be some genetic variation within the *Acremonium coenophialum* isolates, and the endophytes from *Poa autumnalis* might not be *Acremonium coenophialum* as is currently viewed.

E. Random Amplified Polymorphic DNA Analysis

Polymerase Chain Reaction (PCR) is a new procedure to replicate DNA sequences selectively and repeatedly. Specific DNA sequences can be amplified by PCR from the reaction mixture which contains genomic DNA, arbitrary primer, *Taq* DNA polymerase and dNTP. The *Taq* DNA polymerase is a heat resistant DNA polymerase which can stand the high temperature (95°C) during the step of DNA denaturing, and keep its activity at high temperature (up to 72°C). In the reaction, only the DNA sequences between the two binding

points of the used arbitrary primers can be amplified during the cycles of DNA denaturing, annealing and extension (Figure 1.1 and William 1990). Now PCR has been broadly used in a broad area, such as DNA sequencing, DNA amplification and DNA polymorphism studies.

Because the different genomic DNA have different binding sites of primers, the genetic variation can be revealed by the number and length of amplified products of polymerase chain reaction which may be phylogeneticaly conserved or individual-specific (Caetano-Anolles et al., 1991). By utilizing this technique, Random Amplified Polymorphic DNA (RAPD) analysis has been developed recently to detect genetic variation of genomic DNA.

RAPD is a very sensitive, fast and reproducible method. PCR only requires a very simple procedure of DNA extraction. For PCR, DNA can be barely purified and the DNA concentration in the reaction mixture can be as low as on the level of 30 pg (Welsh and McClelland, 1990). This makes it possible to study genomic DNA of a single cell (Lee and Taylor, 1990). Highly reproducible DNA polymorphisms can also be obtained within very broad range: from ng to ng. Within only 2.5 hours, genomic DNA can be tremendously amplified DNA for 35 cycles of PCR(Yu and Pauls, 1992).

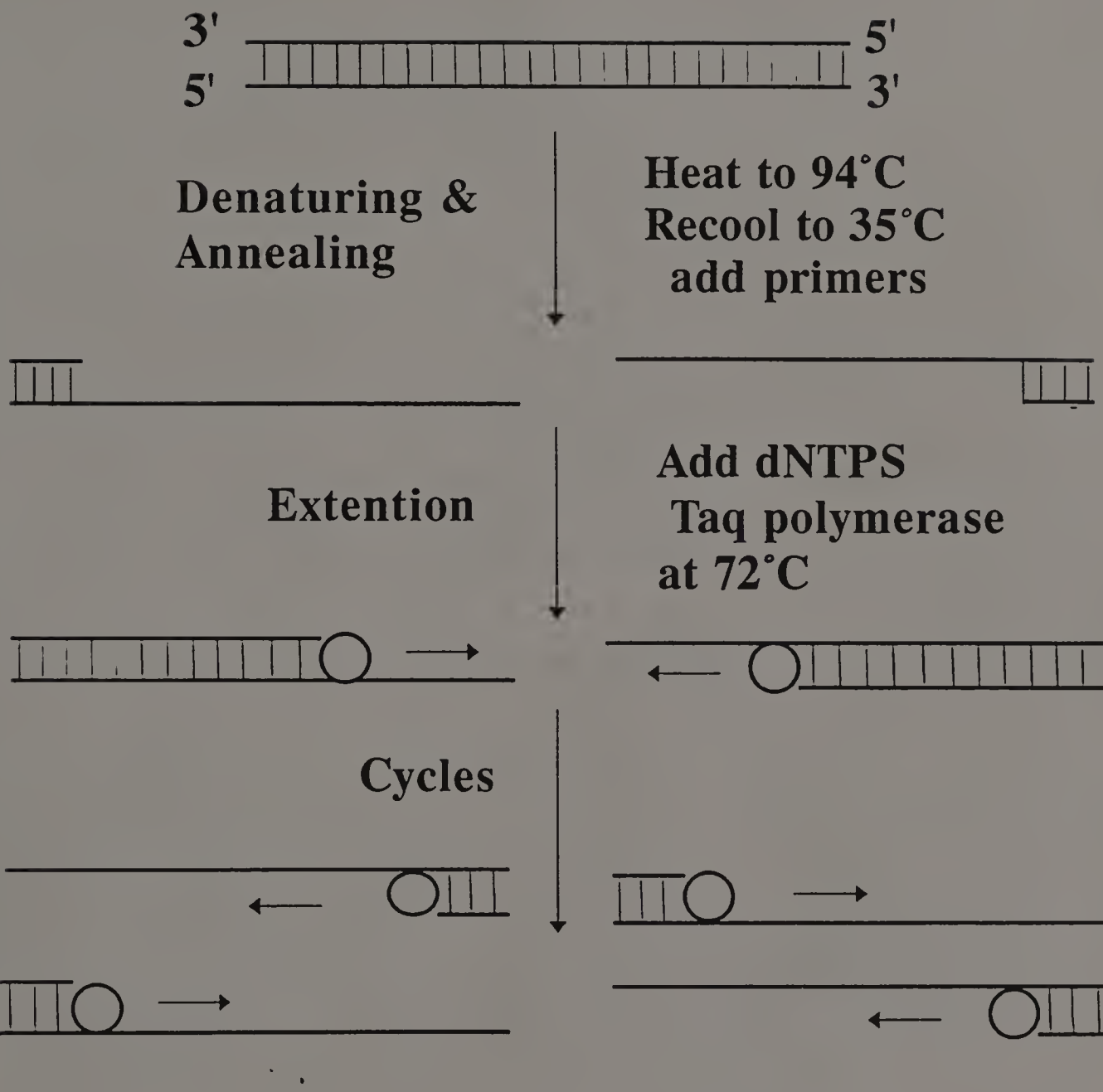

Figure 1.1. The Polymerase Chain Reaction (PCR). Primers are extended by *Taq* DNA polymerase between the primers' two binding position on a DNA molecule. DNA sequence between these two primers is being amplified.

RAPD analysis has been applied to studies of genomic DNA from rabbit, monkey, dwarf Bermuda grass, soybean (Caetano-Anolles et al., 1991), and man, corn and bacterial strains (William et al.,1990). Variation of fungi have also been successfully identified by RAPD analysis (Bruns et al, 1990; Cenis, 1992; Crowhurst et al., 1991; Meyer et al., 1991; Goodwin and Annis, 1991).

CHAPTER II

RAPD ANALYSIS ON FUNGAL DNA VARIATION

A. Introduction

Acremonium coenophialum from different cultivars of tall fescue (*Festuca arundinaceae*) and from *Poa autumnalis* may be different biotypes since they show variation in morphological characters in liquid and semi-solid culture (Noble 1991), production of ergot alkaloid (Bacon, 1988; Bacon and Siegel, 1988; Porter et al., 1979), and isozyme pattern (Leuchtmann and Clay, 1990). To determine if *Acremonium coenophialum* biotype truly exist, and vary in potentially desirable characteristics for biocontrol, it is necessary to determine the genetic variation.

At present, random amplified polymorphic DNA (RAPD) analysis is widely applied to genome mapping, gene tagging and population studies (Goodwin and Annis, 1991; and Williams et al., 1990). Genetic variation can be revealed by the number and length of DNA sequence amplified by Polymerase Chain Reaction (PCR) with arbitrary primers (Caetano-Anolles et al., 1991). This technique has been successfully applied to the identification of genomic DNA and investigation of the DNA variation in man, plant

(Williams et al., 1990), mammals, bacteria (Caetano-Anolles et al., 1991), and fungi (Canis, 1992). It is a simple, fast, reliable, and inexpensive technique.

The objectives of this research were to determine the genetic variation among the *Acremonium coenophialum* endophytes by RAPD analysis.

B. Materials and Methods

Acremonium coenophialum fungal endophyte was isolated from leaf sheaths of their respective hosts and their DNA was isolated form cultured mycelium and were studied by RAPD with random primers.

1. *Acremonium coenophialum* Isolation from Leaf Sheathes

The following endophyte infected grasses were planted in ten 4" pots for each species, and maintained in greenhouse:

a. *Poa autumnalis*;
b. 'Rebel II', tall fescue (*Festuca arundinacea*);
c. 'Titan', tall fescue (*Festuca arundinacea*);
d. 'shenandoah', tall fescue (*Festuca arundinacea*).

The leaf sheaths of *Poa autumnalis*, 'Titan', 'Rebel II' and 'Shenandoah' tall fescue were surface sterilized with full

strength Chlorox (5.25% sodium hypochlorite) with 1.25 ml/L Tween-20 (Bacon, 1988) and then rinsed in sterile distilled water. Sheathes were cut into 1.5 cm segments before placing in petri dishes with Cornmeal-Malt-Medium (CMM, see Appendix A). After 4 weeks of maintenance in the dark at 25°C, mycelium growing from leaf sheaths became visible.

2. *Acremonium coenophialum* Culture.

a. Semi-Solid culture: The fungal isolated were transferred on Cornmeal-Malt-Medium, and maintained in the dark at 25°C.

b. Liquid culture: Each clonies of *Acremonium* from the semi-solid medium was cut into small pieces (about 3 mm x 3 mm) and placed into separated 250 ml flask with 50 ml M102 liquid medium (Appendix A). Liquid cultures were maintained at room temperature on a gyratory shaker at 200 rpm (Bacon, 1988). After cultured in M102 liquid medium for three weeks, fungi were harvested for DNA isolation.

3. Fungal Endophyte DNA Extraction and Purification.

DNA were extracted from three isolates of *Acremonium coenophialum* endophytes from each of 'Rebel II', 'Titan' and

'Shenandoah' tall fescue and *Poa autumnalis* respectively, by the following procedure.

a. DNA Extraction: Five grams fresh weight mycelium of each *Acremonium* isolate in M102 liquid culture was pelletted by centrifugation. The mycelium was ground in 15 ml of ice cold extraction buffer (Appendix B) and filtered through two layers of miracloth. The material on the miracloth was scraped off into a 50 ml polypropylene centrifuge tube with 15 ml nuclei lysis buffer (Appendix B), mixed gently before added 5 ml 5% sarcosyl, and incubated in 60°C water bath for 20 min. 10 ml chloroform/octanol (24:1) was added and gently mixed by 10 - 15 inversions. After a 10 min centrifuge (5000 rpm), the aqueous (top) phase was transferred into a new 50 ml polypropylene centrifuge tube. 16 ml isopropanol was added to the tube and mixed by inversion until the DNA came together. The DNA was hooded out with a bent pasteur pipet, washed in cold 70% ethanol three times and dissolved in 1.5 ml microcentrifuge tube with 1.0 ml of TE buffer (Appendix B) over night.

b. DNA purification: 10 ml chloroform/octanol (24:1) was gently mixed with the DNA solution. After 10 min centrifuge (5000 rpm), the aqueous phase was pipetted off and mixed with 650 ul isopropanol. Then DNA were hooded out, washed and resuspended in 1 ml TE buffer as above.

c. Estimation of DNA concentration: Isolated DNA samples were loaded to a 0.8% agarose gel with several different concentrations of uncut DNA as reference marks. Electrophoresis was run in TAE (Appendix C) buffer at 100 V for one half hour. The gels were stained with ethidium bromide and viewed under UV light (360 nm). The DNA concentration was estimated by comparison of band brightness between sample DNA and standard uncut Lambda DNA. According to the results of the electrophoresis, the isolated DNA were diluted into 50 ng/ul concentration and stored at 4°C. The concentration of diluted DNA were tested again by the same method.

4. Random Amplified Polymorphic DNA (RAPD) Analysis.

After the DNA concentrations were optimized, the amplified polymorphic DNA analysis were carried out on all DNA isolates with 20 random primers (Kit D from Promega, Appendix D), and the results were tested by RAPD analysis on broad ranges of DNA concentration.

a. Polymerase chain reaction (PCR): The reaction mixture was prepared for each polymerase chain reaction according to Table 2.1, and were added into a 0.5 ml

microcentrifuge tubes and covered with 20 ul mineral oil (Sigma).

Table 2.1 Reaction mixtures for PCR.

	Volume (ul)	Final Concentration
Sterilized H_2O	4.8	
x 10 Buffer*	2.5	
100 mM Tris pH 9.0		10 mM
500mM KCl		50 mM
1% X-100 Triton		0.1%
$MgCl_2$* (25mM)	2.0	2.0 mM
dATP* (5mM)	2.5	0.5 mM
dCTP* (5mM)	2.5	0.5 mM
dGTP* (5mM)	2.5	0.5 mM
dTTP* (5mM)	2.5	0.5 mM
Primer* (10uM)	0.5	0.2 uM
Taq DNA polymerase* (5 U/ul)	0.2	0.04 U/ul
Sample DNA	5.0	
Total	25 ul	

* Promega reagent.

The amplification reactions were carried out on a Cetus Perkin-Elmer thermal cycler for 45 cycles of the following conditions:

94°C for 1 min (denaturing)
35°C for 1 min (annealing)
72°C for 2 min (extension)

The reaction mixtures were then stored in the microcentrifuge tubes at 4°C before electrophoresis analysis.

b. Electrophoresis: Amplification products were mixed with 5 ul blue juice (Appendix C) and loaded on 1.4% agarose

gel with 3 ug 123 bp DNA ladders (GIBCO BRL) as markers. The electrophoresis were run in TBE (Appendix C) buffer at 100 V for 3.5 hours. The gels were stained with ethidium bromide (2.5 ug/ml) and viewed under UV light. The primers which amplified some DNA isolate differently from other isolates were picked up for further studies.

c. RAPD analysis with various DNA concentration: To confirm that the differences of amplification patterns among the endophyte DNA isolates could be constantly obtained under a broad range of DNA concentration, three purified DNA replicate from each kind of host grasses were mixed equally, and the RAPD analysis were carried out with five different DNA concentration: 5 ul of about 0.063, 0.25, 1, 4 and 16 ng/ul of the purified DNA samples were added into the PCR mixture respectively. Then the DNA were amplified with those selected primers by the same protocol above.

C. Result

1. *Acremonium coenophialum* Isolation from Leaf Sheaths

On medium *Acremonium coenophialum* endophyte from leave sheath of *Poa autumnalis* became visible in about 2 weeks, but in about 5 weeks for tall fescue endophytes. It was the

same as the results obtained by Noble (1992).

2. *Acremonium coenophialum* Culture

Because *Acremonium* endophytes have very low growth rate, they can not competed with the contaminating fungus. The contaminated *Acremonium* endophytes isolates could be found and eliminated very easily.

The results from both of the solid and liquid culture of *Acremonium coenophialum* consistent with the results obtained by Noble (1991):

a. Solid Culture: Although all the *Acremonium* endophytes grown very slow, the *Acremonium coenophialum* isolates from *Poa autumnalis* had higher growth rate than those from tall fescue (Figure 2.1). No significant differences in growth rate were noticed among *Acremonium* endophyte isolated from 'Titan', 'Rebel II' and 'Shenandoah' tall fescue.

b. Liquid Culture: The mycelial colonies of the *Acremonium* form *Poa autumnalis* were always larger, but fewer in number compared with those from tall fescue (Figure 2.2).

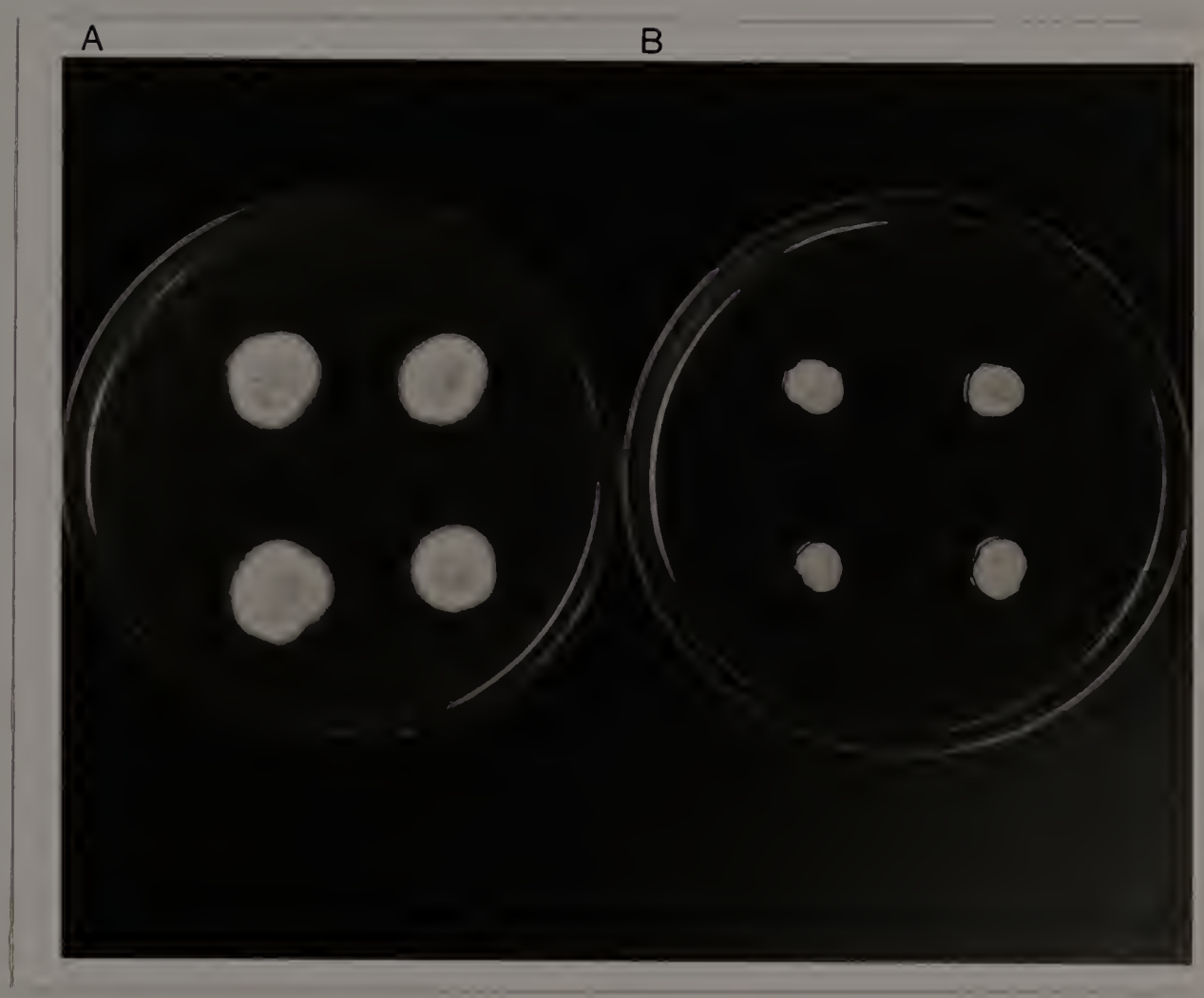

Figure 2.1. The *Acremonium* (A) from *Poa autumnalis* and (B) 'Titan' tall fescue. Pictures were taken three weeks after transfer onto CMM semi-solid medium.

(A)

(B)

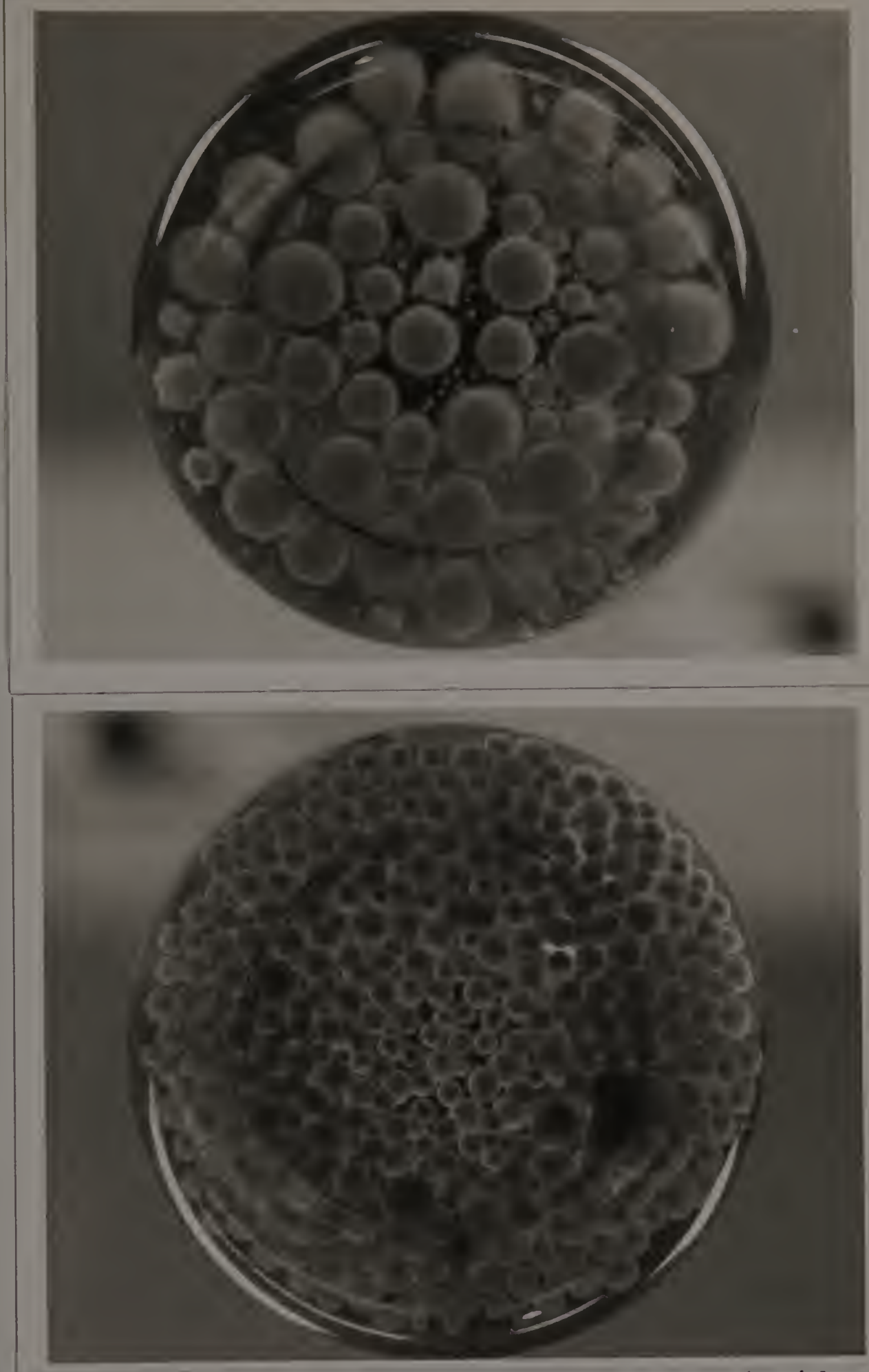

Figure 2.2. *Acremonium coenophialum* in M102 liquid culture. *Acremonium coenophialum* from (A) *Poa autumnalis* and (B) 'Titan' tall fescue, four weeks after transfer from CMM semi-solid medium into M102 liquid medium.

3. Extraction and Purification of Fungal Endophyte DNA

DNA was extracted and purified from mycelium growing in M102 liquid medium. DNA was resuspended in 1.0 ml TE buffer after extraction and purification. DNA concentration was around 90 ~ 100 ng/ul according to the results from DNA concentration gel. The DNA yields from 5 g of fresh weight mycelium were about 90 ~ 100 ug. Then the purified DNA were diluted to 50 ng/ul (Figure 2.3), and stored at 4°C.

4. RAPD Analysis

a. Optimized DNA concentration: According to results of the random amplified polymorphic DNA analysis for the broad ranges of DNA concentration with primer #2 and 13, the optimized DNA concentration of 1 ng/ul was selected for RAPD analysis of all DNA polymorphism. At this concentration, RAPD analysis for most of the DNA isolates could result in sharp band patterns.

b. Polymorphism of DNA isolates: At about 1 ng/ul of DNA concentration, all DNA isolate were tested by PCR with 20 different primers (Kit D, Promega; Appendix D) separately, and very stable DNA polymorphism were obtained.

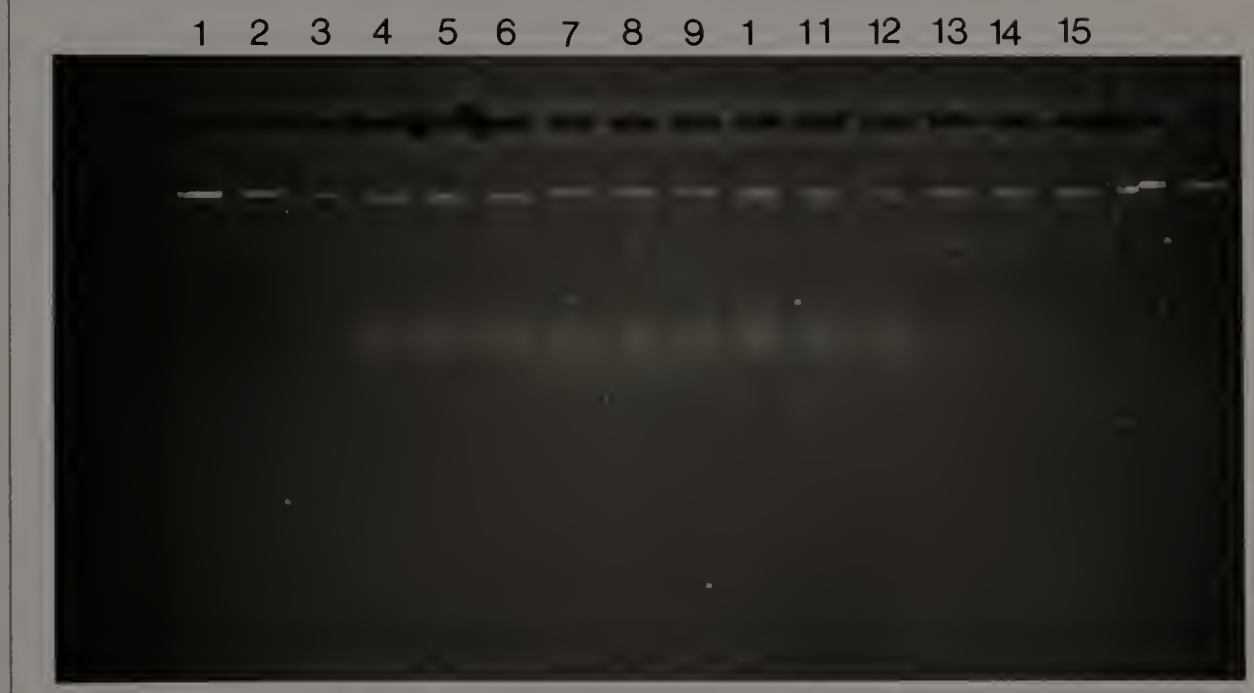

Figure 2.3. Concentration Gel.
Lane 1, 2 and 3 are 100, 50 and 25 ng/ul uncut Lambda DNA standard respectively. Lane 4-6, 7-9 10-12 and 13-15 are purified DNA isolates from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively.

The polymorphism of endophyte DNA from *Poa autumnalis*, 'Titan', 'Rebel II' and 'Shenandoah' tall fescue were shown in Figure 2.4, 2.5, 2.6 and 2.7 respectively. According to the results of the RAPD analysis, 13 primers gave amplification products for all the DNA isolates. They are primer #1, 2, 3, 4, 5, 7, 8, 11, 13, 15, 16, 18 and 20. The other 7 primers (#6, 9, 10, 12, 14, 17 and 19) did not amplify the DNA of any endophytes. With these tested primers, all endophyte DNA from the same host grasses had the same DNA polymorphism.

For the purified DNA from *Poa autumnalis* endophyte (Figure 2.4), the number and length of the amplification products varied among the primers. The number of the amplification products varied from 2 (primer #20) to more than 15 strands (primer #11), and the length from about 300 bp (primer #11) to more than 3,000 bp (primer # 8).

For the purified DNA isolates form tall fescue (Figure 2.5, 2.6 and 2.7), the number of the amplification products varied from 2 to more than 15 strands among the primers. The length of these strands varied from 260 bp to more than 3 kb.

Acremonium from *Poa autumnalis* had different DNA polymorphisms from those isolated from tall fescue in 10

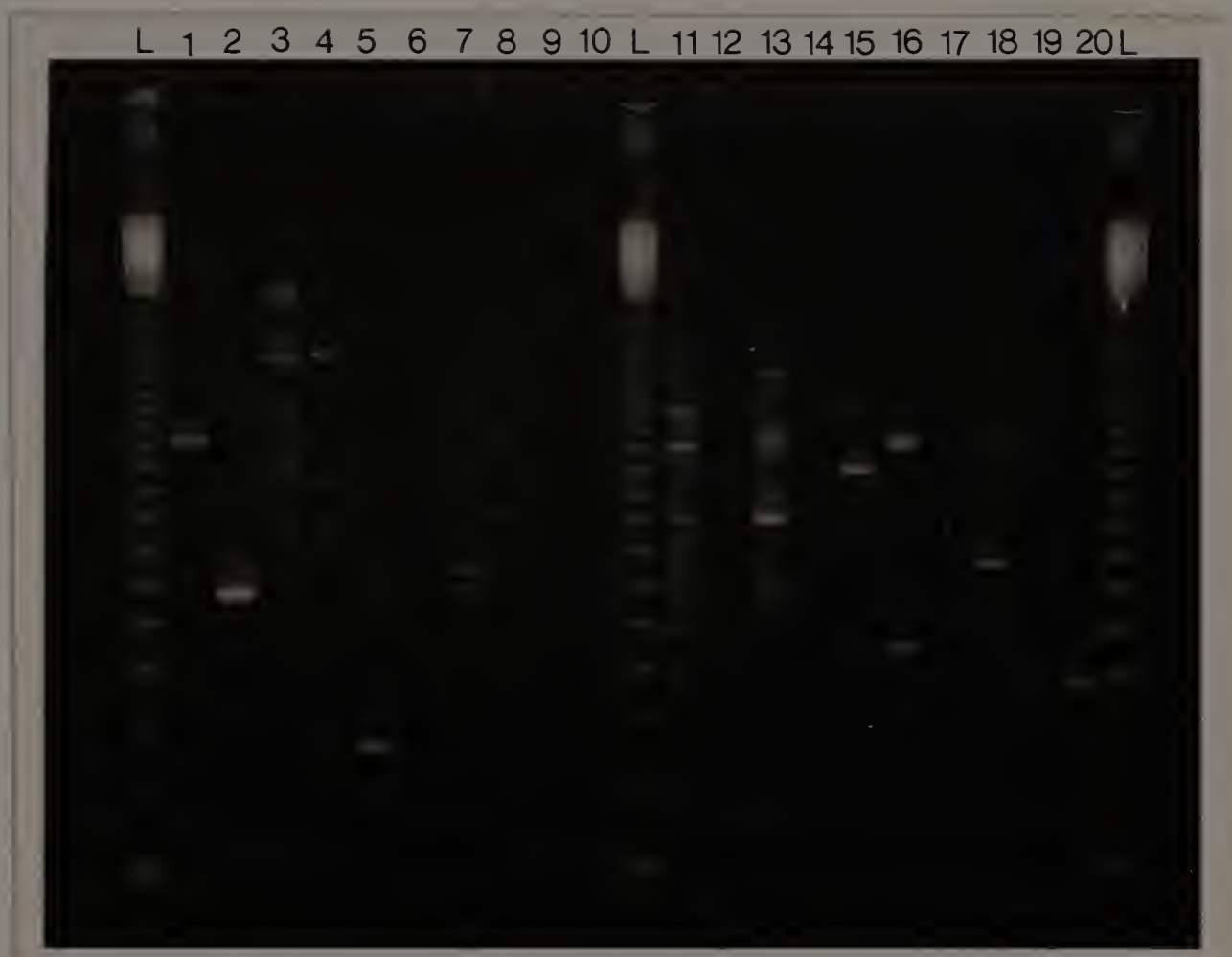

Figure 2.4. DNA polymorphism of *Acremonium* from *Poa autumnalis*.
Lane 1 - 20 are the primers # 1 - 20 respectively. Lane L is the 123 bp DNA ladder. 13 primers (#1, 2, 3, 4, 5, 7, 8, 11, 13, 15, 16, 18 and 20) gave amplification products.

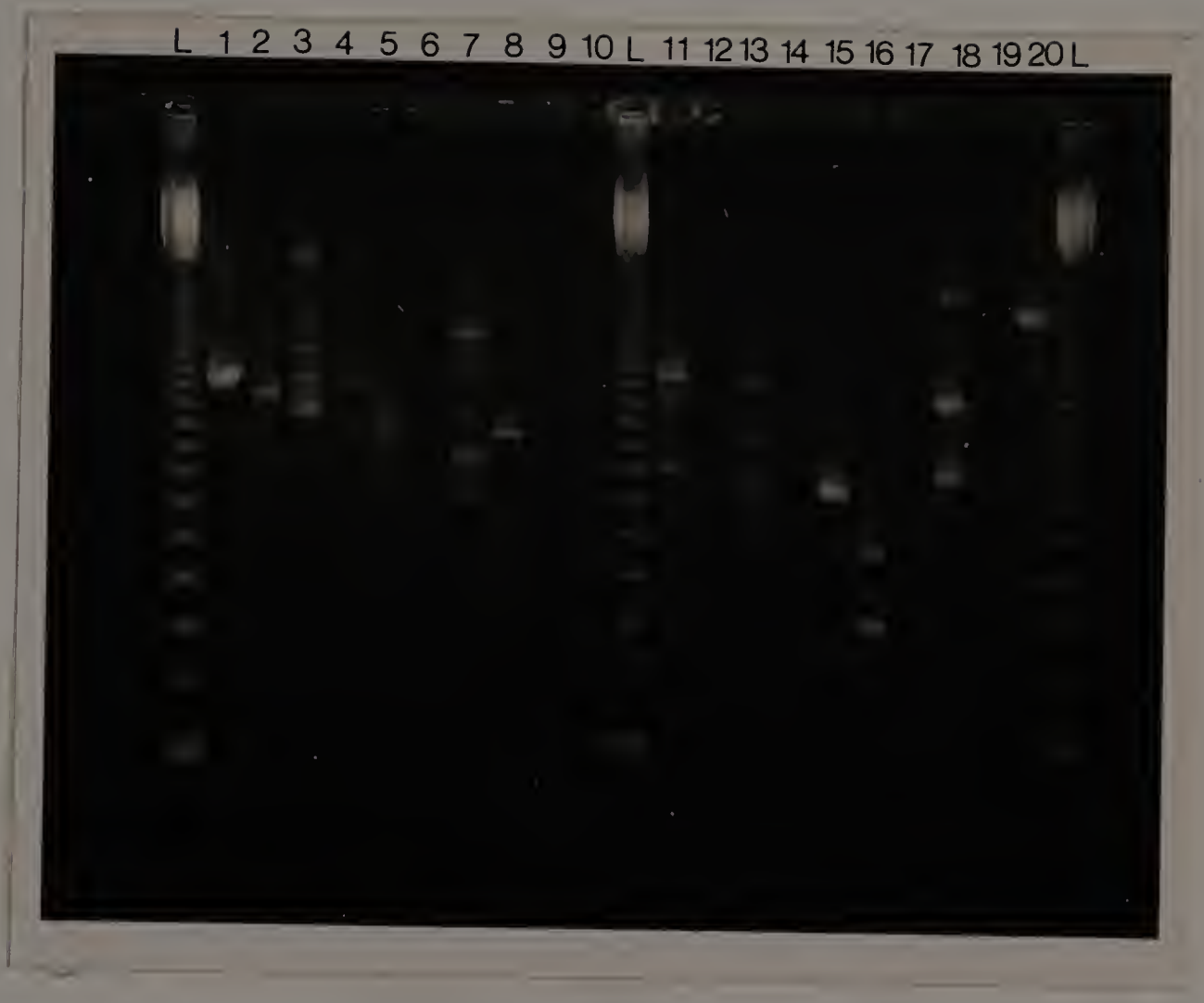

Figure 2.5. DNA polymorphism of *Acremonium coenophialum* from 'Rebel II' tall fescue.
Lane 1 - 20 are the primers # 1 - 20 respectively. Lane L is the 123 bp DNA ladder. 13 primers (#1, 2, 3, 4, 5, 7, 8, 11, 13, 15, 16, 18 and 20) gave amplification products.

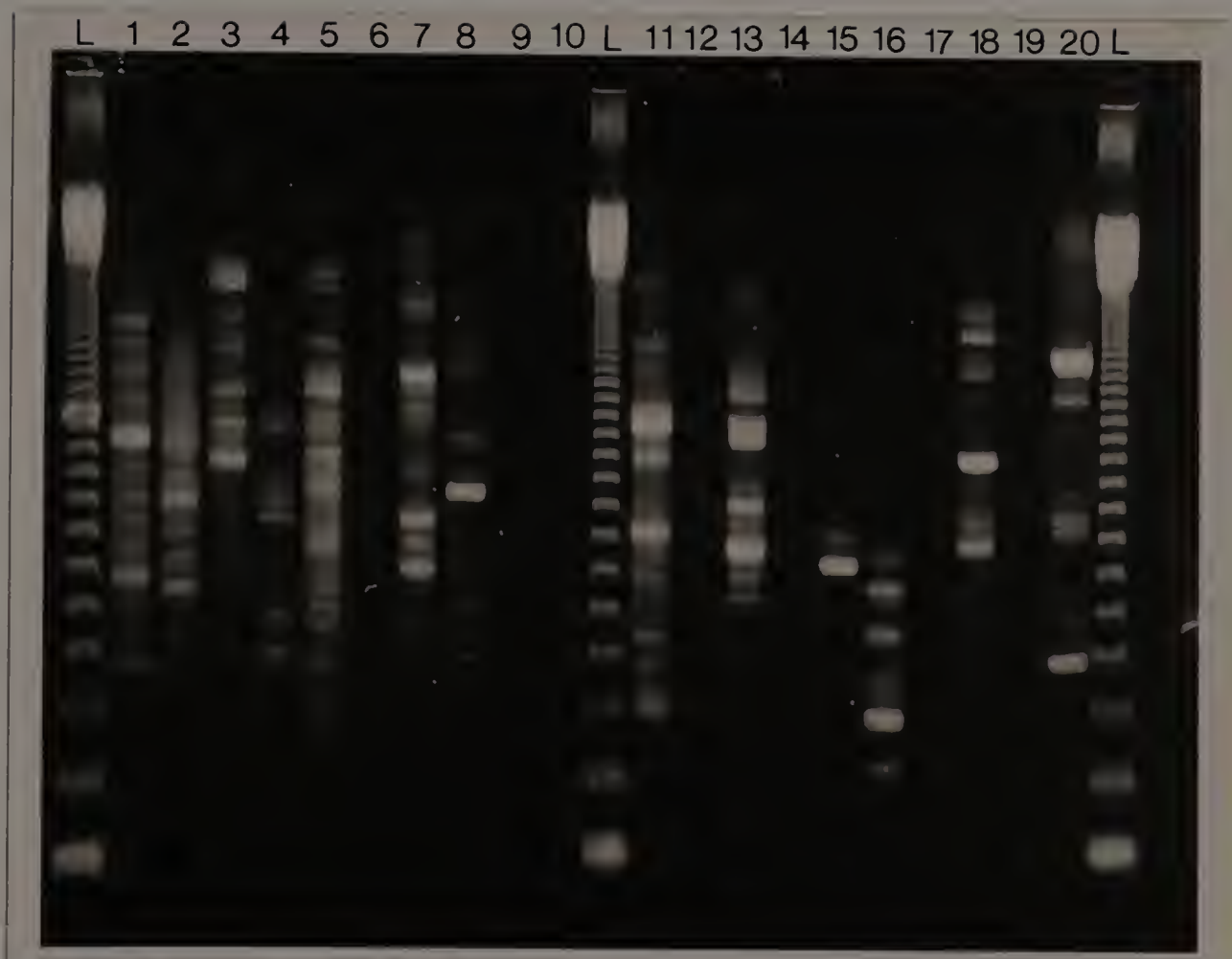

Figure 2.6. DNA polymorphism of *Acremonium coenophialum* from 'Titan' tall fescue.
Lane 1 - 20 are the primers # 1 - 20 respectively. Lane L is the 123 bp DNA ladder. 13 primers (#1, 2, 3, 4, 5, 7, 8, 11, 13, 15, 16, 18 and 20) gave amplification products.

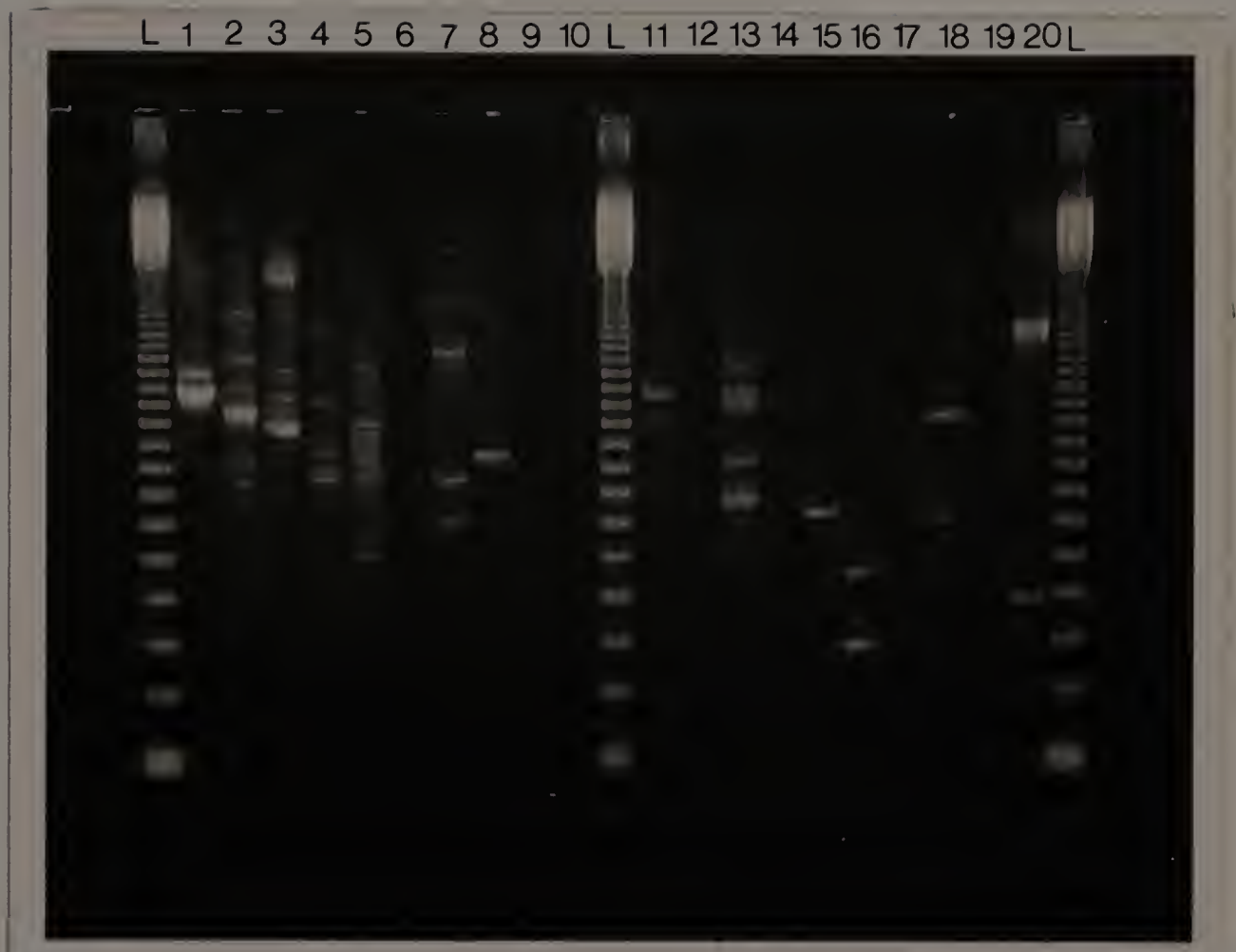

Figure 2.7. DNA polymorphism of *Acremonium coenophialum* from 'shenandoah' tall fescue.
Lane 1 - 20 are the primers # 1 - 20 respectively. Lane L is the 123 bp DNA ladder. 13 primers (#1, 2, 3, 4, 5, 7, 8, 11, 13, 15, 16, 18 and 20) gave amplification products.

Primers (#2, 5, 7, 8, 11, 13, 15, 16, 18 and 20). No variation was found among the DNA polymorphism of *Acremonium coenophialum* isolated from 'Rebel II', 'Titan' and 'Shenandoah' tall fescue.

These results shown that no genetic variation were found among the *Acremonium* from the same host grasses and among the endophyte from tall fescue cultivars. But, the DNA polymorphism of the *Acremonium* from *Poa autumnalis* were significantly different from those of tall fescue endophyte. To confirm these results, RAPD analysis were carried out under the broad range of DNA concentration (0.063 ~ 16 ng/ul).

c. RAPD Analysis in a Broad Rang of DNA Concentration: According to the above results, Primer #2, 5, 8, 11, 15, 16 and 18 were selected for the RAPD analysis in broad ranges of DNA concentrations. The tested concentration of purified DNA isolated were 0.063, 0.25, 1, 4 and 16 ng/ul. The results show that the patterns of DNA polymorphism from each DNA mixture were very stable among the tested concentrations in PCR, and the differences among the *Acremonium* were constantly obtained.

With primer #2 (Figure 2.8), very strong bands were obtained from PCR amplification for all endophytes DNA. A

700 bp DNA segment was strongly amplified from the DNA of *Poa autumnalis* endophyte. In contrast, the DNA from tall fescue endophytes, primer #2 strongly amplified the 1200 bp DNA segment, while the 700 bp DNA band was very weak, in RAPD analysis at all DNA concentrations.

DNA polymorphism amplified with primer #5 (Figure 2.9) shown that only a 320 bp DNA was the amplified segment of DNA from *Poa autumnalis* endophytes while many large DNA sequences (550 ~ 2100 bp) were amplified from the DNA of tall fescue endophyte. Compared with *Acremonium* DNA from the other tall fescue cultivars, the polymorphism of the *Acremonium* DNA from 'Shenandoah' did not have the 550 bp band. This was the only difference found among the DNA polymorphism of *Acremonium coenophialum* from tall fescue.

Many DNA segments were amplified with primer #8 (Figure 2.10). These segments have different length from 0.5 ~ 2.5 Kb. However, the endophyte form *Poa autumnalis* has two more amplified products than the endophyte from the other hosts: they are 0.6 and 1.3 kb segments. The endophyte from tall fescue have a strong 1.1 kb amplified product while the endophyte form *Poa autumnalis* do not.

Five DNA segments were amplified with primer #11 by PCR. They all have very strong 0.9 and 1.4 kb bands. However

the endophyte from *Poa autumnalis* has a particularly strong band at 550 bp and does not have the 370 bp which appears in DNA polymorphisms in tall fescue endophyte.

For DNA from all endophytes, only two segments were amplified with primer #15 (Figure 2.12), however, the amplified segments (1.0 and 1.2 kb) of the DNA of *Poa autumnalis* endophytes were significantly larger than those of tall fescue endophyte DNA (750 and 900 bp).

Two sequences (350 and 700 bp) were strongly amplified with primer #16 in all DNA of tall fescue endophytes (Figure 2.13). But in the polymorphism of endophyte DNA from *Poa autumnalis* endophytes the 350 bp band was missed.

Primer #18 (Figure 2.14) gave numerous strong amplified bands by PCR, and these bands were in the range of 0.5 to 3 kb. The DNA polymorphisms between the endophyte from tall fescue and from *Poa autumnalis* were totally different from each other in the range of 1.0 to 3 kb.

From all these results, no significant variation was found in the endophyte from the same host grasses. *Acremonium* isolated from *Poa autumnalis* had significant differences from *Acremonium coenophialum* from tall fescue in both of their morphological and genetic characters.

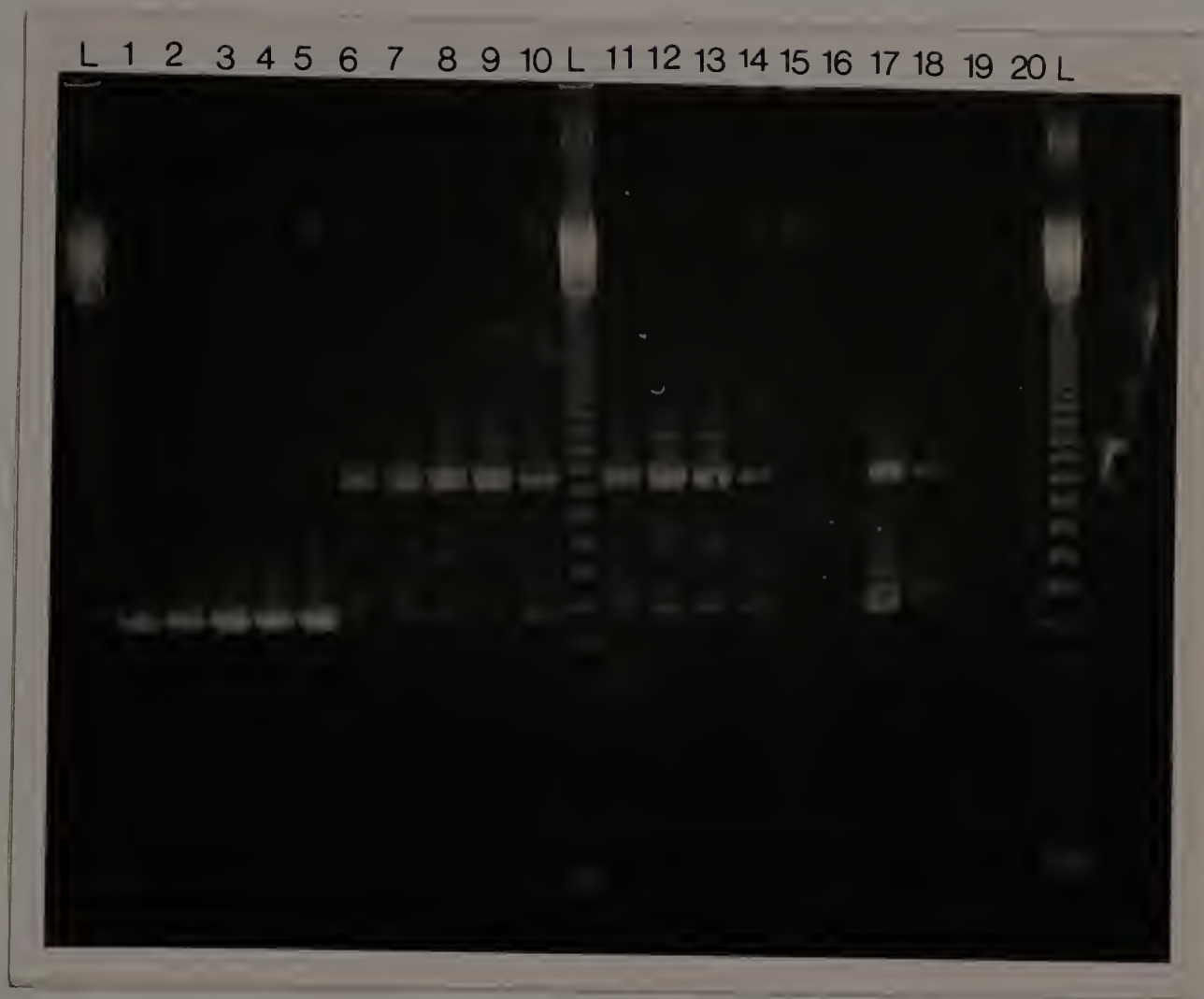

Figure 2.8. RAPD Analysis with Primer #2.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

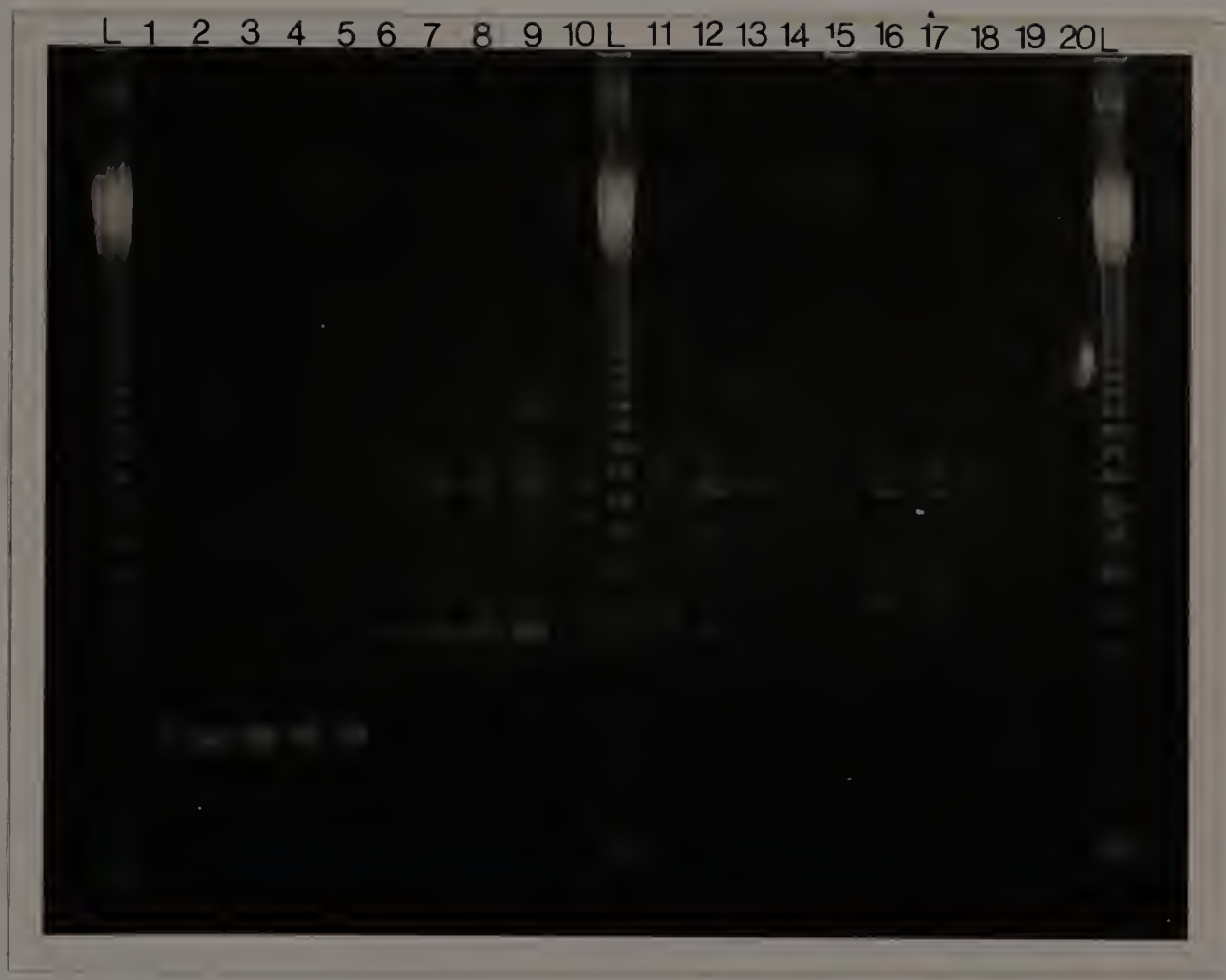

Figure 2.9. RAPD Analysis with Primer #5.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

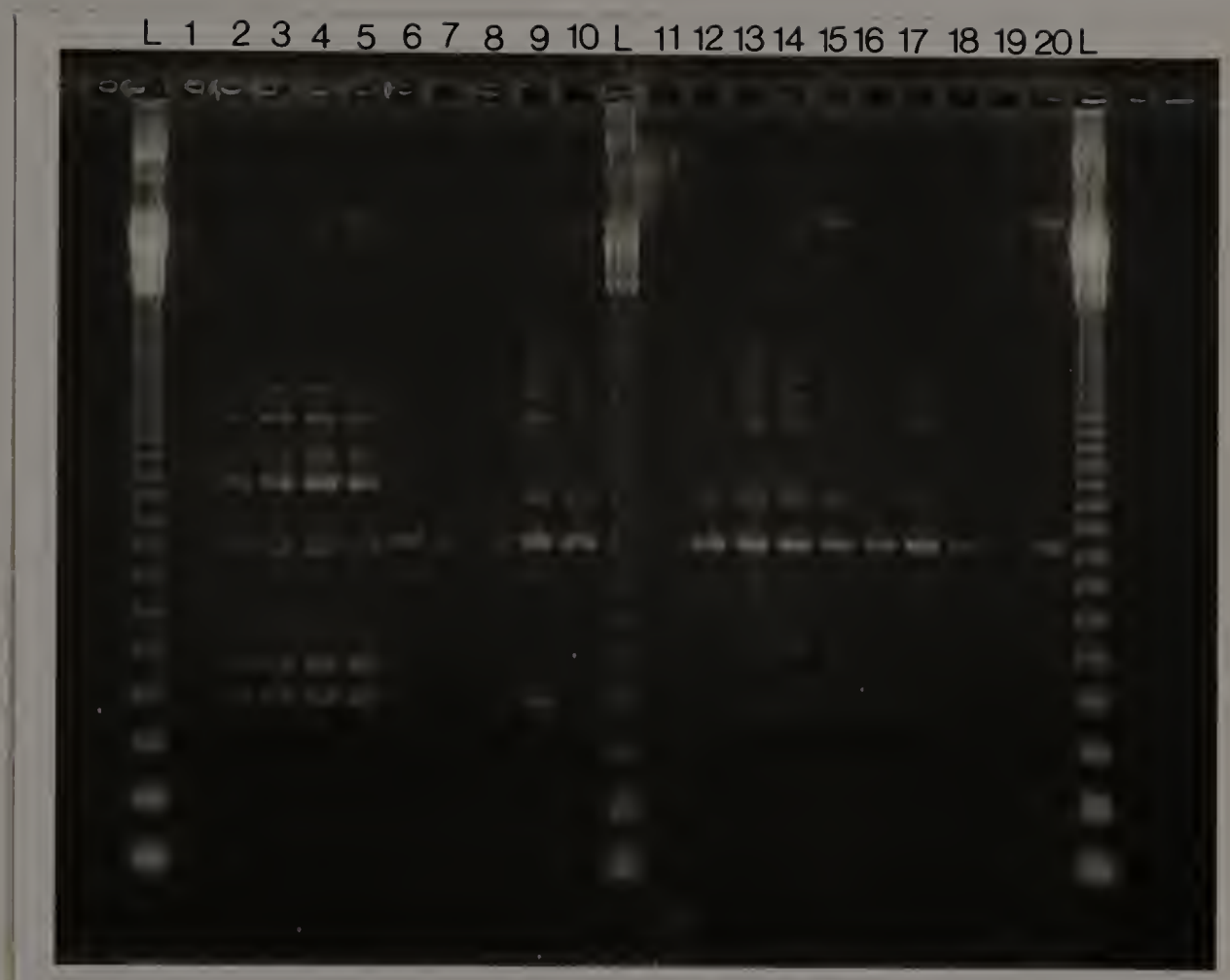

Figure 2.10. RAPD Analysis with Primer #8.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

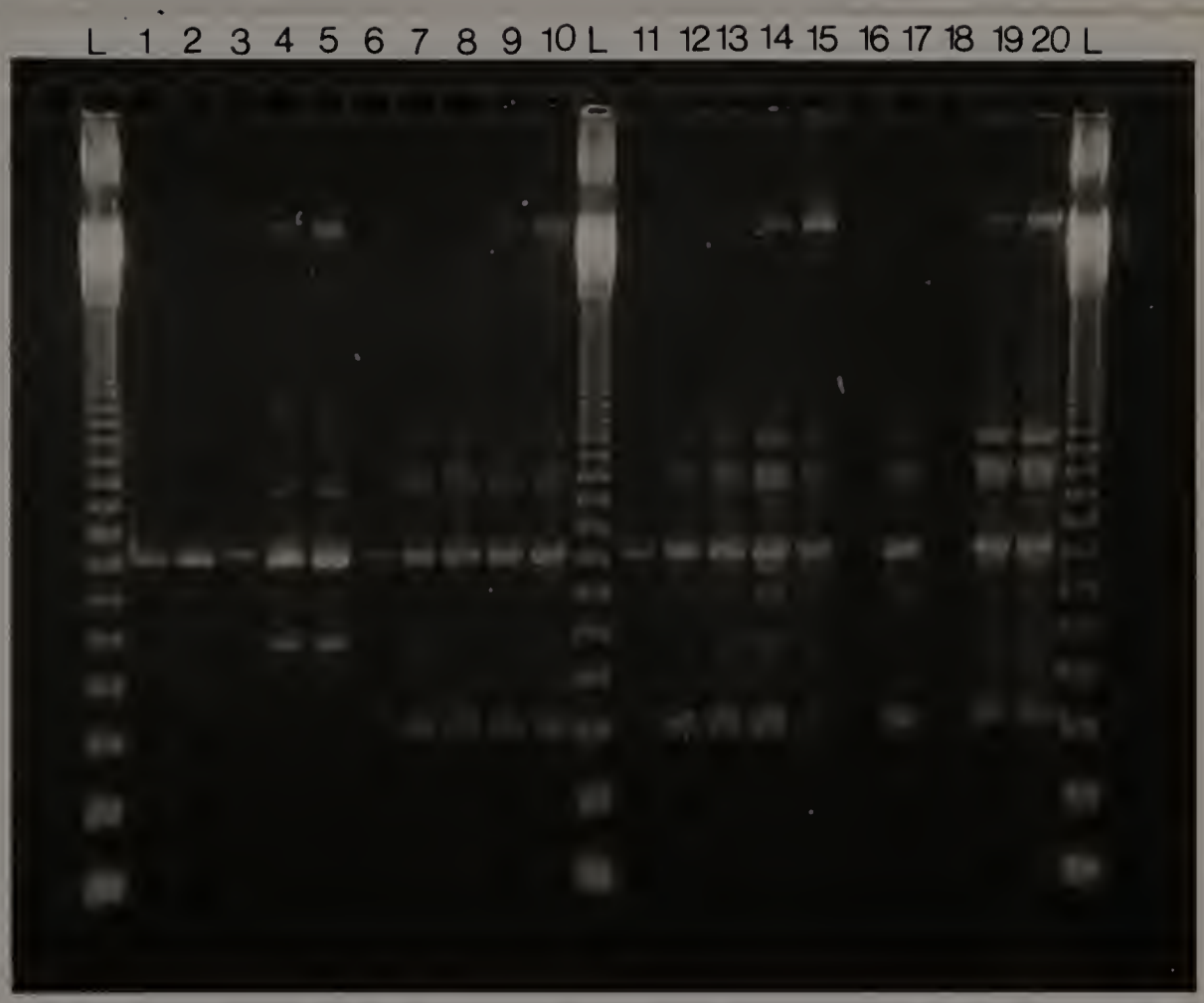

Figure 2.11. RAPD Analysis with Primer #11.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the Acremonium DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

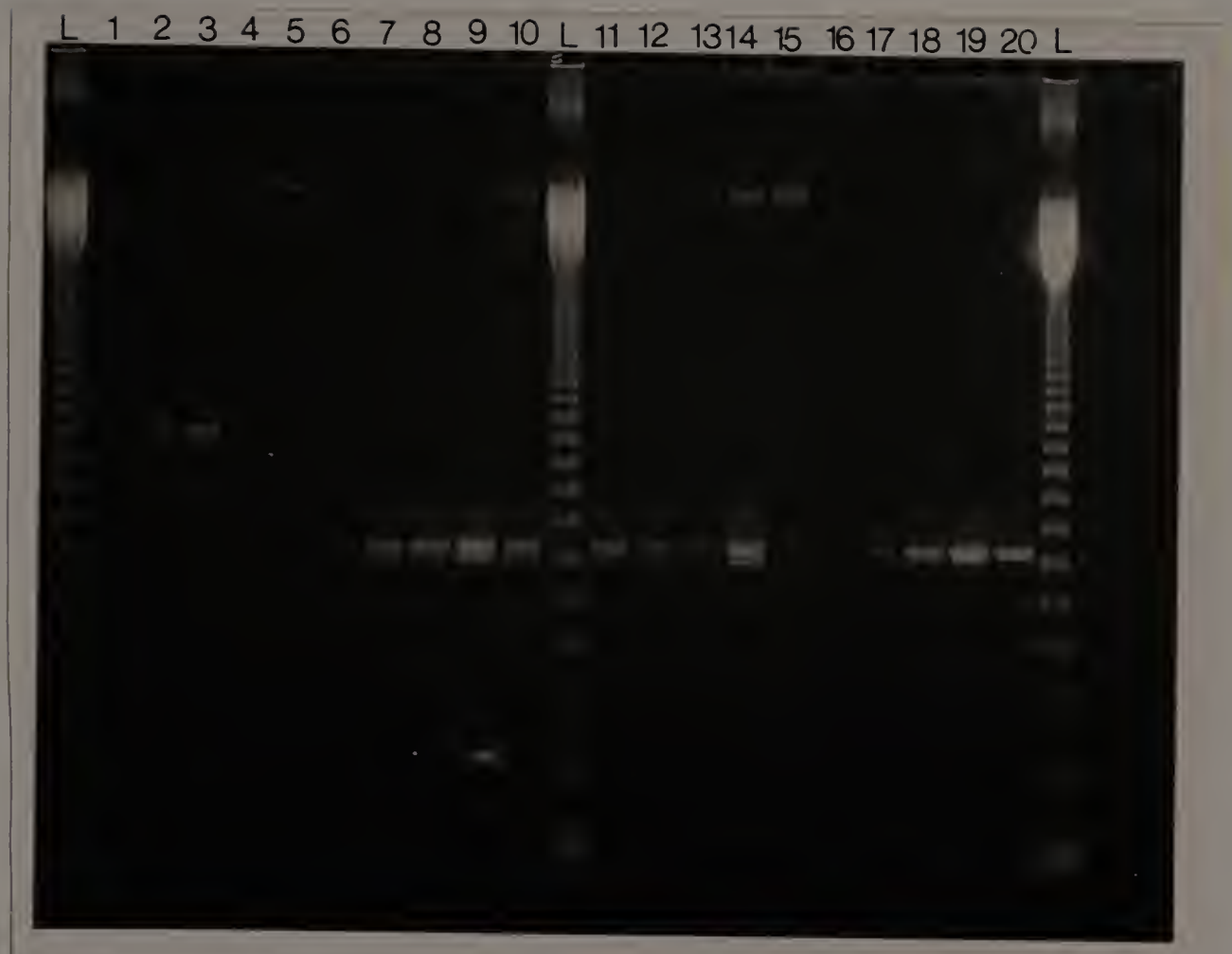

Figure 2.12. RAPD Analysis with Primer #15.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

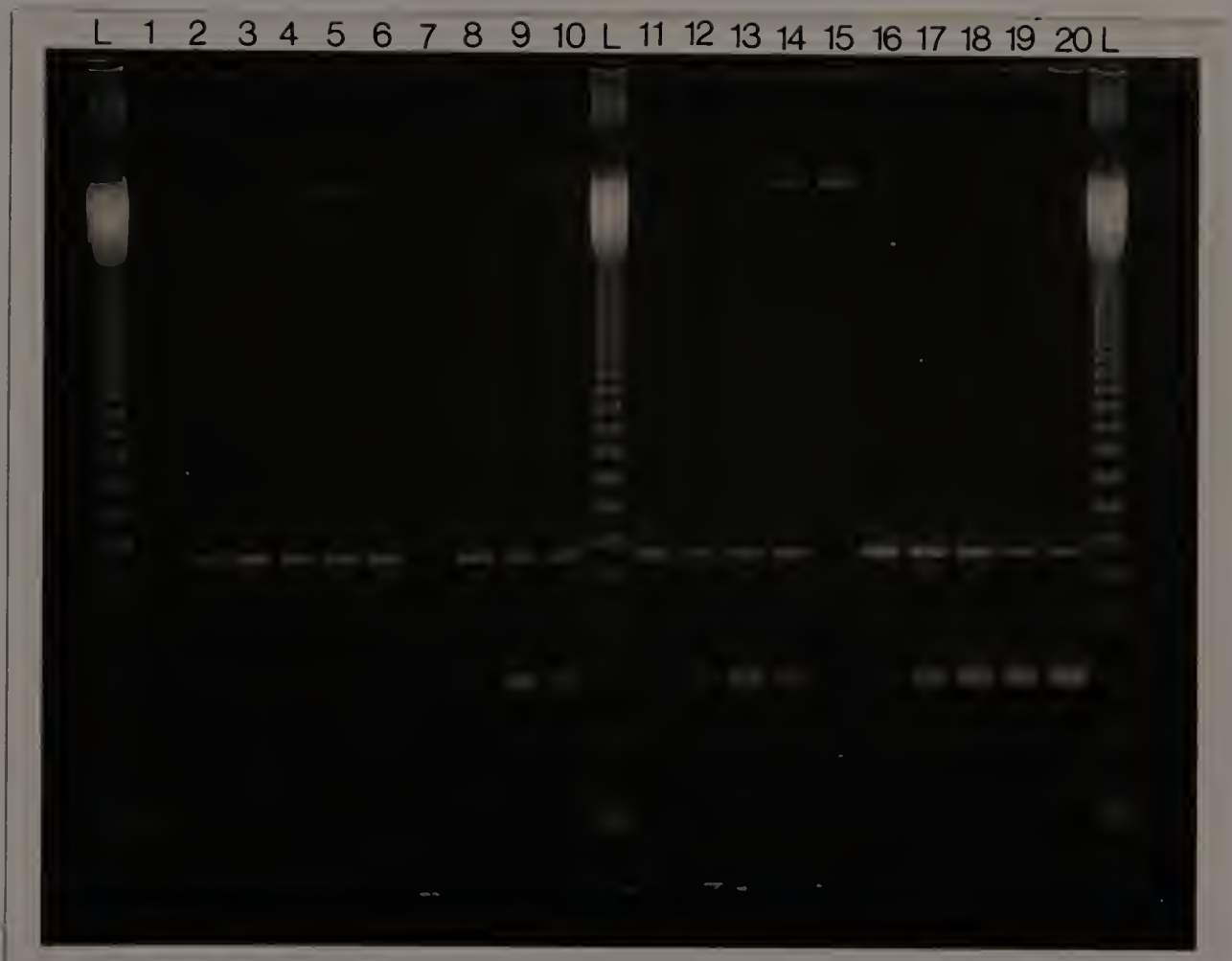

Figure 2.13. RAPD Analysis with Primer #16.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

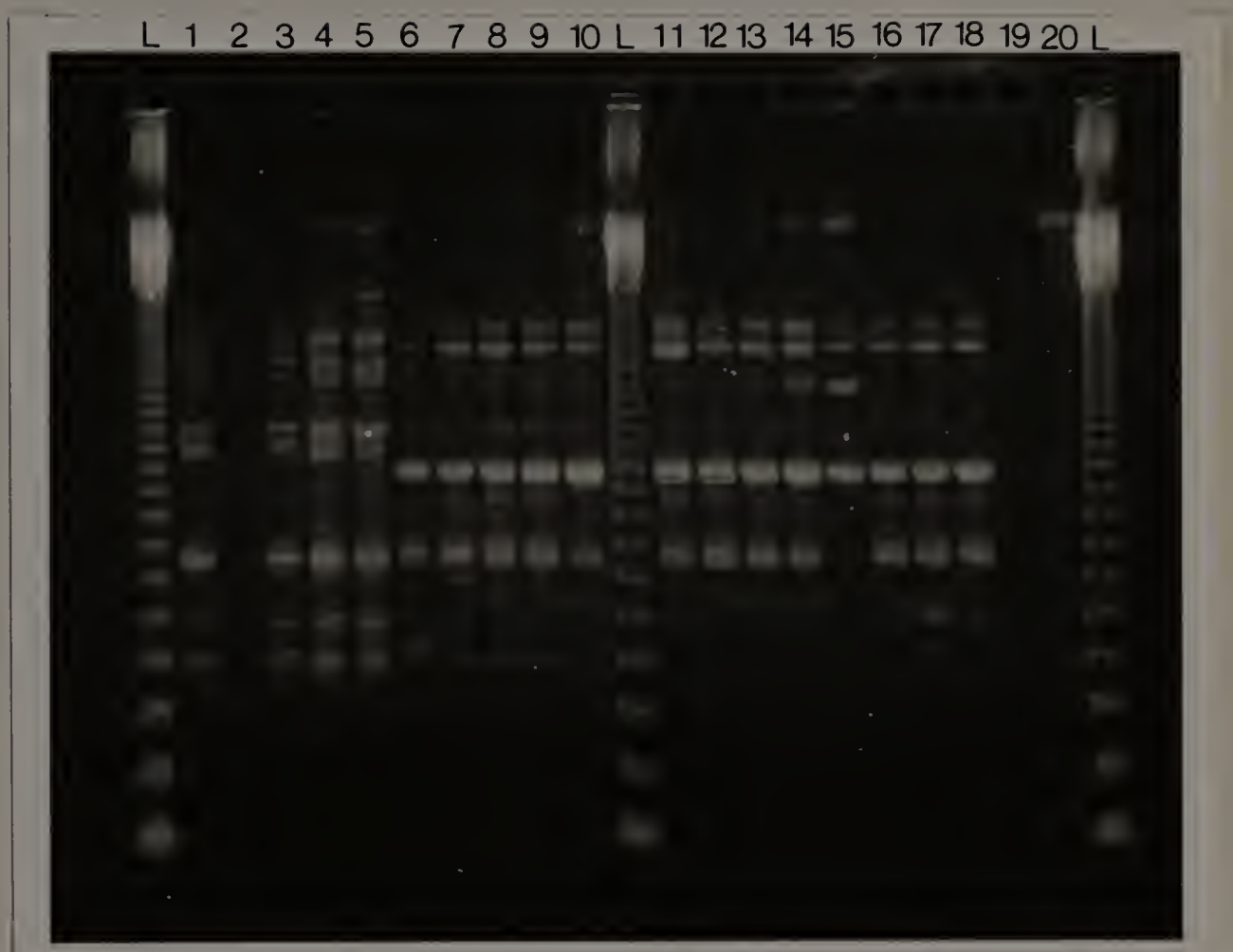

Figure 2.14. RAPD Analysis with Primer #18.
Lane 1 ~ 5, 6 ~ 10, 11 ~ 15 and 16 ~ 20 were the *Acremonium* DNA from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue respectively. The concentrations of each DNA sample were 0.063, 0.25, 1, 4 and 16 ng/ul from the lift to the right. Lane L was the 123 bp DNA ladder.

Acremonium coenophialum from tall fescue had the same morphological characters and DNA polymorphisms, except one single difference between the endophyte from 'Shenandoah' and from other two tall fescue cultivars.

D. Discussion

Morphological variation was shown between *Acremonium coenophialum* isolates from *Poa autumnalis* and tall fescue cultivars. *Acremonium* from *Poa autumnalis* grew faster than those from tall fescue on CMM semi-solid medium. Further in the M102 liquid medium, *Acremonium* from *Poa autumnalis* formed large mycelium balls, in contrast to *Acremonium* from tall fescue which produced numerous small mycelium balls in the same medium. No significant differences were noticed among the *Acremonium coenophialum* from tall fescue cultivars. These results are consistent with those of Nobel (1992).

DNA extraction and purification were very simple and efficient. Five gram fresh weight mycelium balls yielded about 90-100 ug of DNA which is enough for at least several thousands of PCR reaction. The yield, in fact is much higher than the previous report (Cenis, 1992) which use the similar method without DNA purification.

RAPD analysis was very stable and highly repeatable. This indicated that the DNA samples were very clean and the PCR reaction mixtures were efficient.

Since DNA were randomly amplified by PCR, the amplified sequences should locate through out the whole genome of endophyte. The total length of the amplified DNA sequences for each isolate was about 35 kb, indicating that this RAPD study, with 20 random primers, covered a maximum 35 kb area in the endophyte genome. However, because no data has been reported on the size the *Acremonium* genome, the percentage of the coverage of this study is unknown.

The DNA polymorphism of the *Acremonium* from *Poa autumnalis*, 'Rebel II', 'Titan' and 'Shenandoah' tall fescue did not show any genetic variation of the *Acremonium coenophialum* endophyte within the same host grasses.

For the tall fescue endophytes, *Acremonium coenophialum* from 'Shenandoah' did not have the 550 bp band which was amplified from the DNA of endophytes from 'Rebel II' and 'Titan' tall fescue. This was the only differences found in the RAPD analysis with 20 primers. This indicates that possible differences in the production of ergot alkaloids by *Acremonium coenophialum* are not due to wide variance among endophyte isolates, and maybe due to the variance of their

hosts or the interaction between the endophyte and host grasses.

Significant genetic variations were revealed between the *Acremonium* from *Poa autumnalis* and from tall fescue grasses. This variation is consistent with the results of isozyme analysis (Leuchtmann and Clay, 1990), which indicated that the isozyme pattern of *Acremonium coenophialum* from tall fescue cultivars are relatively uniform. For 10 of the 20 primers, the amplification products for the DNA of the endophyte from *Poa autumnalis* were different from those from tall fescue cultivars. The morphological and genetic variation between the endophyte from *Poa autumnalis* is not *Acremonium coenophialum* (Morgan-Jones and Gams, 1992) or even not *Acremonium*.

Further RAPD analysis can be done on *Acremonium* endophyte in many other grasses, such as other tall fescue and perennial ryegrass. This may give us more information about the DNA polymorphisms, variation and classification of *Acremonium*. The variation of ergot alkaloid production of endophyte isolated from tall fescue may or may not due to the genetic differences. Further research also should be done to reveal the relationship between the morphological characters and DNA polymorphisms of the endophytes in grasses.

APPENDIX A

MEDIUMS FOR FUNGAL CULTURE

Table A.1. Cornmeal-Malt-Medium (CMM) for *Acremonium coenophialum* semi-solid culture.

	Game (g)	Final Concentration
Cornmeal agar (Difco)	17	1.7%
Malt extract	20	2.0%
Yeast extract	2	0.02%

They were dissolved in 1,000 ml distilled water (Bacon, 1990). 0.5 ml of 10 mg/ml streptomycin sulfate is added into 1 L autoclave medium.

Table A.2. M102 medium for *Acremonium coenophialum* liquid culture.

	Game (g)	Final Concentration
Malt extract	20	2.0%
Sucrose	30	3.0%
Bacto peptone (Difco)	2	0.20%
Yeast extract	1	0.10%
KCl	0.5	
$MgSO_4$	0.5	
K_2HPO_4	1	

They are dissolved in 1,000 ml distilled water (Bacon, 1990) 0.5 ml of 10 mg/ml streptomycin sulfate is added into 1 L autoclave medium.

APPENDIX B

BUFFERS FOR DNA EXTRACTION AND PURIFICATION

Table B.1. Extraction Buffer (one Liter).

	Games (g)
Sorbitol	64
Tris base	12
EDTA(Na)	1.85

pH is adjusted to 7.5 with 1 N HCl. Add H_2O to one liter. The solution is stored at 4°C. 3.8 g Sodium metabisulfite is added to 20 mM just before the buffer is used.

Table B.2. Nuclei Lysis Buffer (100 ml).

	Volume (ml)	Final Concentration
Tris 1.0 M, pH 8.0	20	0.2 M
EDTA 0.25 M	20	50 mM
NaCl 5.0 M	40	1 M
H_2O	20	

2 g Hexadecyltrimethylammonium (CTAB) is added and dissolved in it. The buffer is store at room temperature.

Table B.3. TE Buffer (One Liter).

	Volume (ml)	Final Concentration
Tris 1.0 M, pH 8.0	10	10 mM
EDTA 0.25 M	4	1 mM

Total volume is adjust to 1 Liter with H_2O.

APPENDIX C

BUFFERS FOR ELECTROPHORESIS

Table C.1. Electrophoresis Buffer.

Buffer	Working Solution	Concentrated Stock Solution (per Liter)
Tris-acetate (TAE)	0.04 M Tris-acetate 1.0 mM EDTA	**50x** 242g Tris base 57 ml glacial acetate acid 100 ml 0.5 M EDTA (pH 8.0)
Tris-borate (TBE)	0.089 M Tris-base 0.089 Boric acid 2 uM EDTA	**5x** 54 g Tris base 27.5 g boric acid 20 ml 0.5M EDTA (pH 8.0)

Table C.2. Gel Loading Buffer x 6 (Blue Juice).

Buffer	Store temperature
0.25% Bromophenol Blue 0.25% Xylene cyanol 30% Glycerol in H_2O	4°C

APPENDIX D

SEQUENCSE OF THE PRIMERS

Table D.1 The Sequences of The primers (Kit D, Promega).

PRIMER #	SEQUENCE	PRIMER #	SEQUENCE
1	ACCGCGAAGG	11	AGCGCCATTG
2	GGACCCAACC	12	CACCGTATCC
3	GTCGCCGTCA	13	GGGGTGACGA
4	TCTGGTGAGG	14	CTTCCCCAAG
5	TGAGCGGACA	15	CATCCGTGCT
6	ACCTGAACGG	16	AGGGCGTAAG
7	TTGGCACGGG	17	TTTCCCACGG
8	GTGTGCCCCA	18	GAGAGCCAAC
9	CTCTGGAGAC	19	CTGGGGACTT
10	GGTCTACACC	20	ACCCGGTCAC

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